Defining regions and rearrangements of the Silene latifolia Y chromosome

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Defining Regions and Rearrangements of the *Silene latifolia* Y Chromosome

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ABSTRACT

We combine data from published marker genotyping of three sets of *S. latifolia* Y chromosome deletion mutants with changed sex phenotypes and add genotypes for several new genic markers to refine the deletion map of the Y chromosome and compare it with the X chromosome genetic map. We conclude that the Y chromosome of this species has been derived through multiple rearrangements of the ancestral gene arrangement and that none of the rearrangements so far detected was involved in stopping X–Y recombination. Different Y genotypes may also differ in their gene content and possibly arrangements, suggesting that mapping the Y-linked sex-determining genes will be difficult, even if many further genic markers are obtained. Even in determining the map of Y chromosome markers to discover all the rearrangements, physical mapping by FISH or other experiments will be essential. Future deletion mapping work should ensure that markers are studied in the parents of deletion mutants and should probably include additional deletions that were not ascertained by causing mutant sex phenotypes.

IN studying the evolution of sex chromosomes, interesting questions include whether and how frequently these chromosomes have been rearranged, and whether this is connected with the evolution of restricted recombination. It is predicted that at least two primary sex-determining genes must have been involved in the evolution of males and females from hermaphrodites: one causing femaleness (a mutation in a gene necessary for male fertility or stamen development, *M*, which should still be present on the Y) and one or more suppressors of femaleness (Su^F genes, sometimes called *GSF*, or gynoecium-suppressing factor) that changed hermaphrodites into males. Additional genetic changes also probably occurred as the Y and X chromosomes evolved. Changes on the Y may include changing allocation toward male functions either of the alleles already carried on the Y or of the gene copies that have transposed onto the Y. In either case, these variants may have detrimental effects on female functions; *i.e.*, they may be sexually antagonistic alleles that would often not evolve if they were not at least partially Y-linked and that select for closer linkage to the sex-determining region of the Y (Charlesworth and Charlesworth 1980; Rice 1987).

The involvement of multiple genes, some with sexually antagonistic effects, predicts that X–Y recombination will become suppressed, and it is well known that sex chromosomes, or parts of chromosomes carrying the sex-determining genes (Ma *et al.* 2004; Peichel *et al.* 2004), are indeed nonrecombining and are thus male specific (reviewed in Skaletsky *et al.* 2003). The mechanism for the recombination suppression, however, is unknown, partly because the best-studied sex chromosome systems, such as those of mammals and Drosophila, evolved long ago and have been largely nonrecombining for long evolutionary periods (Carvalho 2002; Skaletsky *et al.* 2003). Thus it is impossible to determine whether recombination was suppressed due to the following possible causes: (i) effects of recombination modifiers located elsewhere in the genome (specifically affecting recombination between the X–Y pair, as is inferred to be involved in suppressing recombination in the *Neurospora tetraspora* mating-type region Jacobson 2005); (ii) lack of X–Y pairing [perhaps due to divergence in sequence or to the accumulation of repetitive sequences in the Y male-specific region expected for various reasons (Charlesworth *et al.* 1994), causing size differences from the homologous X region]; or (iii) chromosome rearrangements that make pairing impossible or lead to loss of recombinant products (Morgan 1950; Schaeffer and Anderson 2005). Lack of pairing is also involved in *N. tetraspora* (Jacobson 2005), so these different causes of recombination suppression are not mutually exclusive. If inversions are involved in the case of sex chromosomes, either the Y or the X chromosome could be inverted, since either would suppress X–Y recombination.

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession no. EU395622.

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Most changes, however, would be expected on the Y, since selection for recombination suppression operates only in XY males; thus the Y is always present in the sex in which recombination suppression is advantageous, and the X is expected to be selected in this way only about 1/3 of the time.

Rearrangements distinguish the Y and X in mammals, and inversions of the Y continue to occur (Hughes *et al.* 2005; Repping *et al.* 2006). This suggests that these inversions arise, not just in association with recombination suppression, but also late in Y chromosome evolution, when (probably as a *consequence* of absence of recombination with the X) Y chromosomes have accumulated repetitive sequences, including transposable elements (Bachtrog 2003; Skaletsky *et al.* 2003). Repetitive sequences are expected to increase rearrangement rates, and this may explain the palindromic duplications found in the human Y chromosome (Skaletsky *et al.* 2003; Repping *et al.* 2006) and could also contribute to loss of segments of Y chromosomes once genes in these segments have degenerated and become dispensable, along with the evolution of dosage compensation (Orr and Kim 1998).

To test whether recombination suppression between the X and Y chromosomes involved inversions, it is therefore important to examine more recently evolved sex chromosomes, such as those of some plants. Here, we describe new data from the plant *Silene latifolia*, whose Y chromosome is estimated to have begun to diverge from the X ~5–10 million years ago, when recombination ceased between some loci, while other genes stopped recombining much more recently (Nicolas *et al.* 2005; Bergero *et al.* 2007).

Several deletion maps of the *S. latifolia* Y chromosome inferred using molecular markers have tried to localize the sex-determining genes to specific regions of the Y more precisely than by using cytologically determined deletion sizes (Lebel-Hardenack *et al.* 2002; Moore *et al.* 2003; Zluvova *et al.* 2005a). The two arms of the Y are similar in size and distinguishing them has been difficult, but it was recently reported that the pseudo-autosomal region (PAR) is on the *p* arm of the X and the *q* arm of the Y. An AFLP-based genetic map has even suggested that both ends of the sex chromosomes of this species can pair and recombine (Scotti and Delph 2006), although no independent evidence confirming this is yet available; we have assumed that the Y PAR location determined by Zluvova *et al.* (2005a) is correct.

Some physical mapping results are starting to become available to compare with genetic maps of the X chromosome. FISH experiments showed that, in both chromosomes, the *DD44* gene is on the arm opposite from the PAR (Lengerova *et al.* 2003; Zluvova *et al.* 2005a), and recent work using microdissection to isolate the two X arms and test them with several X-linked genes using FISH has confirmed that *DD44*-X and *Slss*-X are on the X*q* arm, along with *SlX4* and *SlX3* (Hobza *et al.* 2007). The genetic map results in *S. latifolia* that include pseudo-autosomal markers (Nicolas *et al.* 2005; Bergero *et al.* 2007) place *DD44*-X close to *Slss*-X, both much less distant from the pseudo-autosomal marker than the genes with large X–Y divergence (genes *SlXY4*, *SlX3*, *SlXY7*, and *SlXY6a*). These two X*q* arm genes should thus be closer to the X centromere than *SlX4* and *SlX3* (Hobza *et al.* 2007).

To date, all Y chromosome deletions have been ascertained by changes in the sex phenotypes of plants. Hermaphrodite deletion strains have been found to have deletions of one arm of the Y (Westergaard 1958; Lardon *et al.* 1999), currently identified as the *p* arm of the Y (Lengerova *et al.* 2003; Zluvova *et al.* 2005a). Deletions causing hermaphrodite flowers can extend up to about half of the arm (Westergaard 1958; Farbos *et al.* 1999; Lardon *et al.* 1999).

Other deletions cause male sterility, and cytogenetic studies show that these can involve either arm of the Y (Westergaard 1958; Farbos *et al.* 1999; Lardon *et al.* 1999), suggesting at least one locus on each arm (Zluvova *et al.* 2007); defects in the latest stages of anther development clearly involve Y*q* arm deletions (see below for more details). However, there are some inconsistencies. For example, *SlY1* is deleted in several hermaphrodite strains, implying a Y*p* location, but also in strains with defects in the latest stages of anther development, requiring a *q* arm location (several other markers are concordant with this gene; see below). Some strains even have deletions of markers believed to be in different Y arms. These data imply deletion of the centromere in some of these sets of deletions and are thus implausible. The explanation for this, and other inconsistencies in the map, has so far been that these strains have undergone two deletions or, for many deletion strains, even more than two, but there is no independent evidence for this from the cytology. Since deletions are expected to be rare events and are indeed found only rarely among the *S. latifolia* progeny plants derived from pollinations using irradiated pollen (Zluvova *et al.* 2007), it is expected that most strains should have single deletions, so that multiple deletions seem unlikely. Data on the distribution of numbers of deletions are scarce, and different plants might differ in this respect, but a study of deletions in a set of 55 chromosomes in γ-irradiated maize (a plant whose genome size is very similar to that of *S. latifolia*), in which the frequency of deletions was very high (29 had one or more deletions), found 18 with single deletions and 11 with more than one [the maximum numbers were high, with one strain having five deletions, and one six (Riera-Lizarazu *et al.* 2000)]. The data are therefore not conclusive, and alternative explanations, not involving multiple deletions, should also be considered and tested.

Markers mistakenly scored as present (*e.g.*, due to genotyping errors, the presence of a band of the expected

size caused by a duplicate elsewhere in the genome, or amplification of a different sequence) will lead to a false appearance of multiple deletions. Similarly, if individual Y chromosome markers or large regions of the Y are duplicated, a deletion can be obscured, since the duplicate(s) of the marker(s) would lead to its being scored as present. These possibilities should not, however, create apparent deletions of markers from both Y arms, which would require failure to detect a marker that is present. Ideally, positive bands should be verified by sequencing. So far, the markers used for deletion maps of the *S. latifolia* Y chromosome have largely been anonymous (including many AFLP markers), and sequences were not verified. However, most of the markers scored were initially identified on the basis of their cosegregation with the Y chromosome. Problems should thus be rare.

It is thus clearly important to examine the *S. latifolia* Y chromosome further and to map the deletions more reliably. Further work with more markers, and specifically with genic markers that can be verified, is needed. Eleven Y-linked genes have now been found (Bergero *et al.* 2007), and we report here new results using a larger set of genes than has previously been possible and the most complete set of deletions, many of which were used in previous Y mapping. We genotyped the new markers and relate the new results to those already published, including all available physical map information. We compare the maps from three different parental strains. Although the results do not yet solve all the puzzles, they add important new understanding and show what will be required to make further progress.

MATERIALS AND METHODS

Sources of the deletions and information about their sex phenotypes: Table 1 shows the sources of the plants studied and the phenotypes by which the deletions were ascertained. We studied as many as possible of the strains previously studied by Lebel-Hardenack *et al.* (2002), Moore *et al.* (2003), and Zluvova *et al.* (2005a). We refer to these as the U and M series. Both are of European origin; the U series are from a plant from an inbreeding experiment using plants from unknown populations, and the M series are from another plant whose origin is also unknown, other than that it is unrelated to the parent of the U plants (Lebel-Hardenack *et al.* 2002). Previous publications refer to these as the "U9 ecotype" and the "MR4x64 ecotype" (Lebel-Hardenack *et al.* 2002; Moore *et al.* 2003). No cytogenetic information is available on the size/positions of these deletions. These sets of deletions are distinct from those studied by Lardon *et al.* (1999) and Farbos *et al.* (1999), for which sizes are available for some deletions. As many as possible of these deletions were also included (referred to here as the French strains). In addition, one spontaneous mutant was found in Edinburgh. The numbers of mutants from these different parents are listed in separate rows in Table 1, and the results in Figure 1 are shown separately for the deletions derived from the different parentals.

The new markers: We analyzed data on 44 molecular markers in total, of which most are the AFLP and other markers previously studied (Lebel-Hardenack *et al.* 2002; Moore *et al.* 2003; Zluvova *et al.* 2005a). Nine of the markers are genes with X and Y homologs: the Y genes are named *SlY1*, *SlY3*, *DD44-Y*, *SlssY*, *SlY4*, *SlCypY*, *SlY7* and two copies of a duplicated gene, *SlY6a* and *SlY6b*. The final four genes (*SlCypY*, *SlY7*, *SlY6a*, and *SlY6b*) are newly identified genes (Bergero *et al.* 2007).

We also included another gene recently identified from a cDNA sequence and named *SlY8*. This is a putative oxygen-binding coding gene, showing homology (76% amino acid identity) with the *Arabidopsis thaliana* locus *At1G69500*. The coding sequence of the *SlY8* cDNA is interrupted by a 74-bp region with no homology with the coding sequence of *At1G69500* and causing a frameshift. The insertion site of the indel corresponds to a splicing site in the *A. thaliana* cDNA, suggesting that the insertion is an intron that is not correctly spliced out in the cDNA. On PCR with primers based on the cDNA sequence, a product was present only in males of mapping families, indicating Y-linkage. *SlY8* was therefore identified as a Y-linked pseudogene. All attempts failed to isolate the X counterpart from female individuals, despite using several primer combinations; this is unlikely to be because of sequence differences, since a homologous PCR product was obtained from the related species *Silene vulgaris*. There is thus no X chromosome map location for this gene. With the previously studied *SlAp3Y* gene, which has no X homolog (Matsunaga *et al.* 2003), our analyses therefore now include a total of 11 genic markers.

Analyses: The markers and sex phenotypes were used to form deletion maps by manipulating columns in an Excel file to minimize the number of chromosomal breaks. As will be explained below, several different orders of markers are compatible with the data. Inversions were inferred by the program GRIMM (Tesler 2002) using possible orderings of the genic markers on the Y.

RESULTS AND DISCUSSION

The Y map and evidence for rearrangements relative to the X chromosome: Data exist for a total of 61 deletion strains (Table 1), but no strains were genotyped for all markers, and no markers were genotyped for all strains (Figure 1). Supplemental Figure 1 shows the complete set of results, and Figure 1 shows the result for just the genic markers, indicating also their order in the genetic map of the X chromosome; two genes, *SlY8* and *SlAp3Y* (Matsunaga *et al.* 2003), have no X copies and are not numbered. Some of the French strains have been previously studied cytologically, and Figure 1B indicates the estimated deletion sizes on the basis of the published data (Farbos *et al.* 1999; Lardon *et al.* 1999). In addition to the markers, Figure 1 shows possible locations of Y-linked genes whose deletion affects sex phenotypes, inferred from co-deletion with our markers. In Figure 1, we indicate the location for the Y-linked Su^F gene suggested by our marker data (see below). The Y also carries a stamen-promoting factor (*SPF*); following Zluvova *et al.* (2007), we have identified *SPF* with M1 on the basis of the fact that the anther defects in *S. latifolia* females occur early in development, resembling the defects in plants with Y chromosome deletions with early stage male sterility. This is good evidence that females lack a functional *SPF* gene, but does not prove

TABLE 1

Sources of the plants studied and the phenotypes by which they were ascertained

	Phenotypes of the strains and nos. of each mutant phenotype			
		Male steriles		
Source of plants	Hermaphrodites	Early	Intermediate	Late
M series	7	4	4	7
U series	11	3	3	3
French strains	5	2	7	4
Edinburgh plant	0	0	0	1
Total	23	9	14	15

that loss of this function was the initial mutation (see below). Figure 1 also indicates the two other Y-linked male fertility factors identified by earlier studies (reviewed by Zluvova *et al.* 2007); deletions in the M2 region cause anther defects later than M1 deletions, and deletions in the M3 region cause pollen sterility.

Although DNA from the M0 plants used to obtain the deletions was not available, it is apparent that one locus, *SlY6b*, is deleted in the Y chromosomes of two of the three strains in which multiple deletions were produced (the parents of the M series strains and of the French strains), since this marker is absent from all these plants, regardless of their sex status (Figure 1, A and B). This locus is absent in about half of all plants in a sample from multiple natural populations (R. Bergero, unpublished data), which was used to establish Y linkage by checking that variants are found only in males (Bergero *et al.* 2007). *SlY6b* is thus informative only in the U series strains and the *Pf5* mutant (Table 1 and Figure 1C). It is thus possible that the arrangements of genes and markers might differ among different Y chromosomes sampled from natural populations, and we therefore examined the different sets of strains separately, as shown in Figure 1.

Even with the new information and new markers, only two regions are well resolved (those deleted in the Y*p* arm in hermaphrodite mutants and in the *q* arm in plants showing late male sterility). Hermaphroditic mutants are deleted for up to 51% of the *p* arm and must be deleted for a female-suppressing (Su^F) gene, one of the two kinds of primary sex-determining genes expected. Even considering only the presumably more reliable genic markers, it is not possible to find an order for the markers such that each strain has only a single deletion. Thus the positions of many markers are not yet certain. Figure 1 shows orders that are supported by the genic markers (and often also by co-deletion of several other markers in multiple strains; see supplemental Figure 1) and that attempt to minimize the numbers of deletions that must be assumed.

Some of the instances of an isolated apparent marker present in a region where deletion is expected might be due to amplification of other genomic sequences, and some isolated results suggesting deletion of a marker could represent other genotyping errors. However, genotyping errors seem highly unlikely for the genic markers, which mostly behave very reliably in our tests. The one exception is the *SlY3* gene, whose published location is not confirmed by our results using different primers for the PCR tests (of 36 strains tested independently, our tests find that this gene is present in three strains where it was previously stated to be absent and absent in three where it was thought to be present by Zluvova *et al.* 2005a; see supplemental Figure 1); thus results for this gene are not useful for reliable inferences (Figure 1 shows only the genotypes newly determined in our study). We outline next some conclusions that can be made, and Figure 2 summarizes the rearrangements that can be inferred.

First, the location of one new marker, the *SlY6a* gene, is probably unchanged from its position on the X. The gene order in the X map, starting from the PAR, is shown on the left in Figure 2. *SlX6a* maps at the opposite end of the X linkage group from the PAR-X, near *SlX4* and *SlX7*, and considerably distal to *SlX3*, on the basis of mapping in a single full-sib family. This location is consistent with previous results from other families that mapped *SlX3* and *SlX4* (Bergero *et al.* 2007) and with the high sequence divergence between the X and Y copies of all these genes, which suggests that they have not been recombining since early in the evolution of the Y chromosome and are part of the oldest evolutionary stratum of the Y. It is also consistent with the *SlY6a* marker being deleted in many hermaphrodite strains of all three series, *i.e.*, with a location close to the Su^F gene, near the end of the Y*p* arm (Figure 1). It has generally been assumed that all hermaphrodite deletions include the terminal region, since one of these deletions was tested for a Y*p* arm terminal marker, the X43.1 repeat, which was found to be absent, showing that the deletion includes the terminal region of the arm. However, our results fit best with some of the mutant strains retaining the most terminal marker *SlY6a*.

Second, the other known genes from this stratum are never (*SlY4*) or rarely (*SlY7* and *SlY3*) deleted in hermaphrodites. This supports the previous inference of

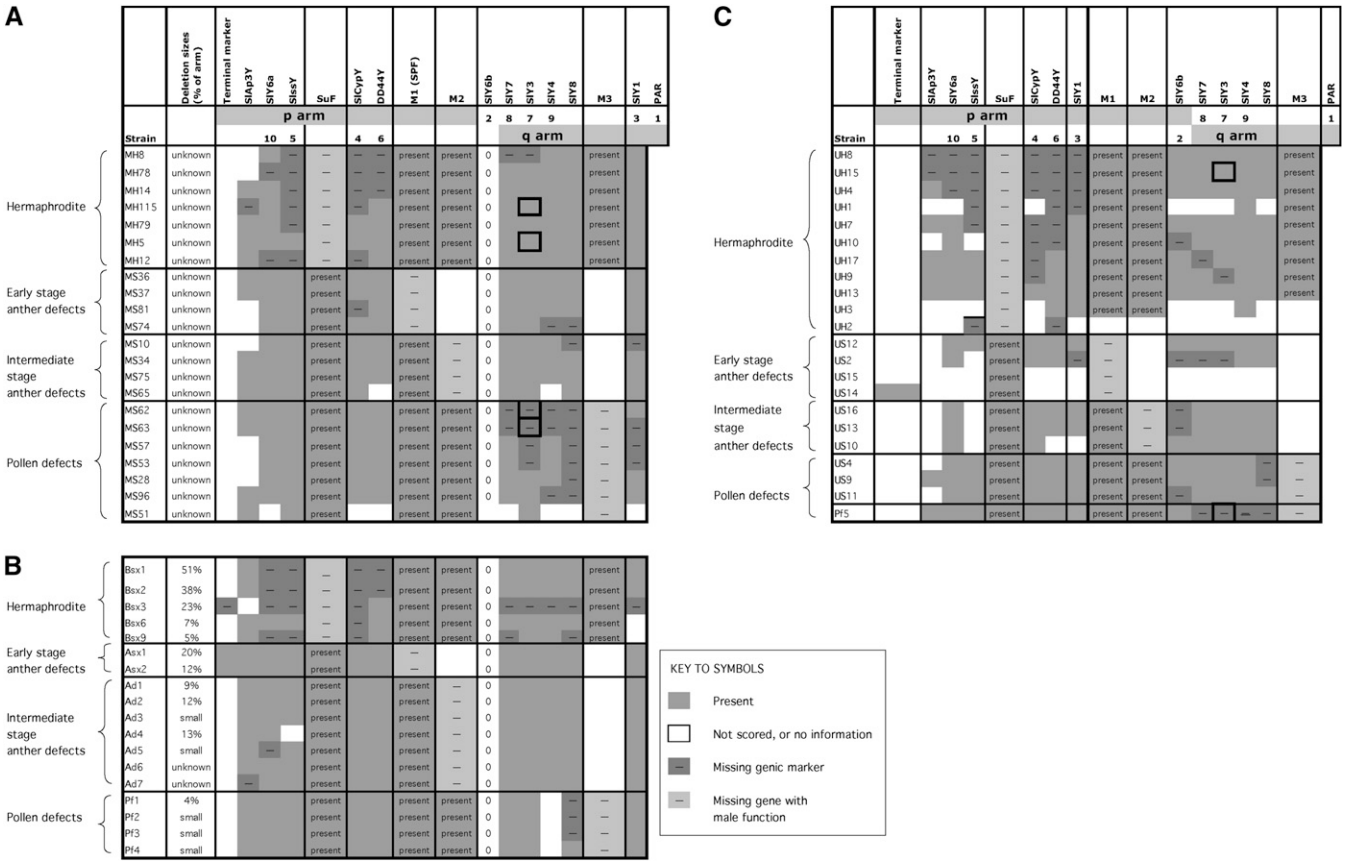

FIGURE 1.—Marker genotyping results from the sets of deletion strains from three different initial M0 plants. (A and B) The M and French sets, respectively. (C) The strains of set U. The sizes of the deletions are known only for some of the French strains, and the column with these data is absent from the other diagrams. In all the diagrams, the pseudo-autosomal region is assumed to be at the right end of the markers on the basis of the Y*p* arm terminal deletion (LARDON *et al.* 1999) clearly mapping at the other end. The genic marker names are shown in boldface type (and the markers are numbered according to their order in the genetic map of the X chromosome, with the pseudo-autosomal region labeled with number 1 and the most distal marker on the other arm of the X, *SlX6a*, labeled number 10 in A–C). The positions of the genes whose deletion affects the flower phenotype are shown as wider columns, and their inferred states (deleted or present) are gray. The position of the *SlY3* gene is uncertain (see text), but is indicated as on the Y*q* arm, where it appears to fit best with either set of genotypes. The markers and strains with differences from the data in ZLUVOVA *et al.* (2005a), in which fewer strains were genotyped, are indicated by boxed cells in the diagrams. Zeros in the column for the *SlY6b* gene indicate that there is no information in plants from these M0 parents, because the parent plant is deleted for this marker (mutants of set M and the French set; see text). Missing data (markers that were not scored in certain strains) are indicated by open cells (in supplemental Figure 1, the states of these cells are inferred, showing that many of them are consistent with other, probably physically close, markers).

major rearrangements between the X and the Y chromosomes (ZLUVOVA *et al.* 2005a; HOBZA *et al.* 2007). Third, in all three sets of strains, in addition to *SlY6a* discussed above, hermaphroditic mutants consistently show deletions of the new marker, *SlCypY*. The *SlCypY* results are consistent with previously published deletion maps, since the X-linked copy of *SlCyp* maps quite near *SlssX* and *DD44*-X (see Figure 2). These three genes have thus probably remained together, and all are now close to the Su^F gene (the Y-linked suppressor of femaleness). *SlCypY* is the first known gene that is almost always deleted in hermaphroditic mutants. Among the 20 hermaphrodite strains in which it could be tested, this marker is deleted in 18 strains (90%), similar to the MK17 marker (deleted in 11/12, or 92% of the genotyped strains; HOBZA *et al.* 2006).

Figure 2 shows an interpretation of the rearrangements necessary to account for these locations. We did not include the *SlAp3Y* gene, because it has been scored in only a few strains and is deleted in very few of them, making its Y location uncertain (it could be located at the distal part of the Y*p* arm, or possibly at the centromere-proximal end); also, it is absent from the X, making it uninformative about rearrangements. It is not yet known whether the transposition of *SlAp3Y* onto the Y chromosome involved a region including other genes or a small region containing *SlAp3Y* only.

The data fit well with the two inversions proposed (HOBZA *et al.* 2007) and the order of the inversions assumed by these authors, although the position of the *SlY3* gene, between *SlY4* and *SlY7*, *vs.* far proximal to both of them on the X map, is accounted for. Figure 2

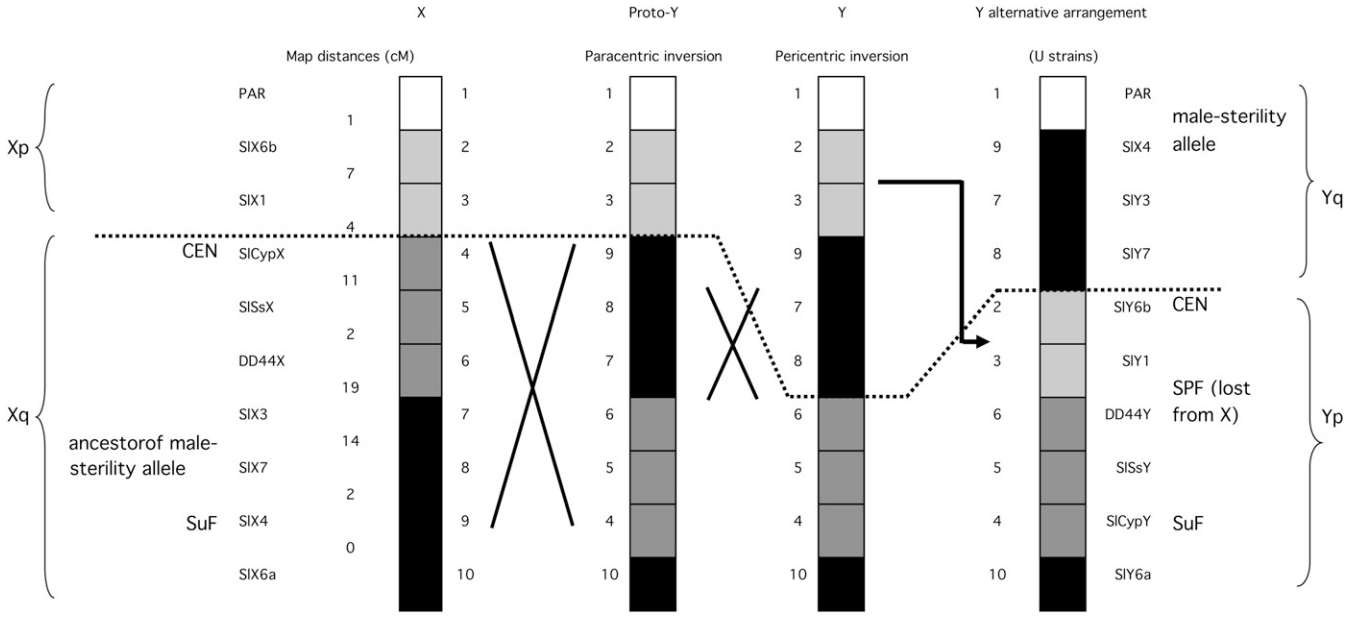

Figure 2.—Interpretation of the results in Figure 1 and supplemental Figure 1, showing the inversions necessary to account for the deletion data. The shading and numerals indicate X–Y divergence levels (black boxes indicate ~20% silent-site divergence, and open boxes indicate the PAR, with no divergence). The version shown assumes that *SlCypX* is on the X*q* arm. If it is located on the X*p* arm, the pericentric inversion would bring it to a position proximal to the centromere of the Y chromosome, relative to the *DD44*-Y gene on the Y*p* arm. Therefore, either *DD44*-Y should be placed distal to the *SlSsY* gene (which does not fit the data as well as the position shown, but requires two further strains to have multiple deletions) or a further rearrangement would be necessary to move *SlCypY* to a more distal position. The right side of the diagram shows the putative alternative arrangement of the Y chromosome in the set of U strains, in which *SlY1* is apparently on the Y*p* arm (see text).

also shows the probable locations of the putative primary sex-determining genes in the initial (X) arrangement and the present arrangement of the Y chromosome and in the intermediate stages (proto-Y chromosomes). It is not certain whether the *SlCypX* gene is on the X*p* or X*q* arm; if the former, further rearrangements would be necessary to move this gene close to the Su^F gene. Although the locations of the *SlY4*, *SlY3*, *SlY7*, and *SlY8* genes are somewhat uncertain, they are clearly not near the Su^F gene and can all be assigned to the Y*q* arm, supporting the previous conclusions about *SlY4* and *SlY3* and inference of a pericentric inversion (Zluvova *et al.* 2005a; Matsunaga 2006; Hobza *et al.* 2007). However, in all three sets of deletion strains two deletions are needed to explain the absence of the *SlY3* and *SlY7* markers in some hermaphrodites. Pericentric inversions are unusual, because they often reduce the fertility of heterozygotes, but such inversions have occurred in Drosophila without reduced fertility and without chromosome pairing (Coyne *et al.* 1993).

The location of the gene(s) whose deletion causes early or intermediate-stage male sterility is very uncertain. Anonymous markers support the cytological evidence that these deletions involve the *Yp* arm (Lardon *et al.* 1999; Lebel-Hardenack *et al.* 2002; Moore *et al.* 2003; Zluvova *et al.* 2005a). If there has indeed been a pericentric inversion moving the *SlY4*, *SlY3*, and *SlY7* genes onto the *Yq* arm, deletions causing early or intermediate-stage male sterility should therefore not involve these markers, since deletions should not cross the centromere. Of the 23 mutants with these phenotypes, however, 5 have possible deletions of these Y*q* gene markers (ignoring *SlY6b*, whose uncertain location is discussed below). This contrasts strongly with the late male-sterility mutations, for which Y*q* deletions are clearly indicated. Some of the apparent deletions of Y*p* markers could represent multiple deletions or genotyping errors, since another discrepancy for these markers is that, despite their putative Y*q* location, 3 of the same mutant class appear to have deletions of Y*p* markers, as do a few hermaphrodite mutants from each set (at least 6 in total). It is surprising that only two markers are regularly deleted in these strains (both anonymous ones; see supplemental Figure 1). Either this is a physically small region, or perhaps it contains few genes.

In the two sets of strains that do not have the *SlX6b* locus (the French strains and the M strains), the order of the genic markers appears to be the same (see Figure 1, A and B). The only puzzle is that the deletion in the French *bsx3* hermaphrodite strain is believed to be smaller than that in *bsx1* and *bsx2*, whereas only the *bsx3* deletion extends to the terminal marker. However, the deletions in *bsx1* and *bsx2* (but not in *bsx3*) include *DD44*-Y (Figure 1), and the size of the region deleted between this gene and the closest male-fertility gene could be large, if the *SlAp3Y* gene (not deleted in *bsx1* and *bsx2*) lies at the distal part of Y*p* arm (see above).

The *SlY6b* gene is present in the U strains, and its location must differ from that of its X homolog. *SlX6b* maps very close to the X pseudo-autosomal marker (Bergero *et al.* 2007), yet its Y-linked homolog seems not to be near the end of the Y*q* arm, but is deleted in three of the five plants with early and intermediate male sterility for which it was genotyped, suggesting a possible location on the centromere end of the Y*p* arm (Figures 1 and 2). The *SlX1* gene is the closest marker to *SlX6b* on the X map and has the lowest X–Y divergence, suggesting that it is closest to the X chromosome PAR, consistent with its location in the M and French strains. In the U strains, however, *SlY1* also appears to map to the Y*p* arm, and several anonymous markers have similar deletion patterns (supplemental Figure 1). Thus, rather than multiple deletions having occurred in the same mutant strains, some of the discrepancies may be resolved if the Y arrangements of the parent strains differ.

Did the Y rearrangements evolve to suppress X–Y recombination? Recombination should have stopped first in the Y region containing the primary sex-determining loci (to prevent the *Su*^{F} and *SPF* genes from recombining). X–Y divergence should therefore be highest in this region; in Figure 2, these genes are black in the diagram of the X chromosome, and the initial arrangement of the Y must have been the same as this. This "stratum" of high divergence should contain the primary sex-determining genes.

In Figure 1, we identified the *SPF* gene with the Y chromosome *p* arm M1 gene, whose deletion leads to early stage male sterility (following Zluvova *et al.* 2007, as explained above). In Figure 2, the X chromosome copy of the gene that mutated is labeled "ancestor of male-sterility allele" because the evolution of females must have involved a mutation ($M \rightarrow m$) in an ancestral allele of a Y gene. Concerning the hypothesis that the *SPF* gene is the ancestor of the male-sterility allele, it is difficult to account for our data, given the current location of M1 near the Y*p* arm centromere, because the genes with high X–Y divergence (black in Figure 2) are almost never co-deleted with the M1 locus (Figure 1). An alternative possibility (Zluvova *et al.* 2005b) is that the initial male-sterility mutation occurred in the ancestral allele of the Y*q* arm M3 gene, whose deletion leads to pollen sterility (and co-deletion of loci that have high X–Y divergence; see Figure 2). To account for the similar stamen development in females and in the class of Y chromosome deletions with early anther defects, another change must subsequently have occurred on the X, perhaps in the M1 gene, or *SPF*, causing anther development to fail (this would not be deleterious in male steriles). If the interpretation is correct that the initial male-sterility mutation was an M3 gene pollen sterility mutation, the genes on the ancestral chromosome that underwent the two initial mutations leading to dioecy in *S. latifolia* could have been located within a small chromosome region, and recombination would have stopped first within this region (black in Figure 2). This is consistent with the "linkage constraint" hypothesized in the first steps of sex chromosome evolution (Charlesworth and Charlesworth 1978). On this interpretation, further selection for suppressed recombination might result from an advantage of preserving X-linkage of the initial pollen sterility allele and the allele that suppressed anther development, since both these would be advantageous in females, but disadvantageous in males.

With either hypothesis for the initial male-sterility mutation, it seems unlikely that the Y rearrangements evolved to suppress X–Y recombination in the initial sex-determining region containing *Su*^{F} and ancestor of the male-sterility allele. Figure 2 shows clearly that the first (paracentric) inversion must have occurred after recombination ceased, since it includes genes of the high divergence stratum and ones with lower divergence. This would imply either that inversions occurred within the highly diverged region (and are not resolved by our mapping) or that recombination was initially suppressed by a mechanism not involving inversions. The second possibility would be consistent with results showing that a deletion causing intermediate-stage male sterility (*Ad7*) also allows X–Y pairing (Zluvova *et al.* 2007). The inversions on the Y chromosome would then be a consequence of suppressed recombination, as discussed in the Introduction.

The evolution of the *S. latifolia* Y chromosome: The proposed inversions are generally consistent with the relative sizes of the X and Y arms, with the pericentric inversion increasing the number of genes on the Y arm carrying the PAR and leaving the other arm as the shorter Y*p* arm. Given the similarity of the *S. latifolia* X chromosome map to that of *S. vulgaris* (Filatov 2005) and the simple relationship of divergence to this genetic map, our results support the previous conclusion that it is the Y, rather than the X, that has been rearranged (Zluvova *et al.* 2005a).

Many problems, however, remain in accounting for the present state of the Y chromosome, particularly if different Y arrangements exist in this species, as our results suggest. It is difficult to account for the results from the deletion strains with only the two inversions proposed, and further rearrangements may have occurred. If *SlY1* and *SlY6b* are on the Y*p* arm in some plants, this cannot be due to another pericentric inversion in the Y arrangement of Figure 2 (since that would bring other markers to that arm, inconsistent with other data from the strain in question, and would leave the Y*p* as a very small arm), but could have arisen by an independent pericentric inversion in the proto-Y arrangement. It is tempting also to consider the possibility that the large physical size of the *S. latifolia* Y may be partly due to duplications, and these might explain some of the puzzles in the mapping results. FISH experiments could resolve these questions, if large-enough genomic

clones can be obtained. If duplications of X-linked genes are not found, this will suggest that the large size of the Y must be due to insertion of nongenic sequences, such as repetitive sequences, for which there is some evidence (Kejnovsky *et al.* 2006a,b), or to transposition of genes or genome regions from chromosomes other than the X, such as the event that added *SlAp3Y* (Matsunaga *et al.* 2003). So far, most known multicopy genes on the *S. latifolia* sex chromosomes, including the *SlXY6a* and *SlXY6b* genes (Bergero *et al.* 2007), are present on both the X and the Y (Matsunaga *et al.* 2005).

Although some of the gene orders are not yet firmly determined, and the map may change as more markers are added to the Y and X, the *SlY6a* marker should be useful in testing for terminal deletions. The lack of correspondence between deletion sizes and numbers of markers deleted suggests that the regions in which we have markers may not represent the whole of the Y. If there are Y regions that do not contain any genes affecting the flower sex phenotype, deletion of these regions will not be ascertained by the approach so far used. There thus might be regions in which deletions of our markers could occur, but which are absent from our map, making it harder to interpret the data. There could also be Y regions with low gene density, and thus a low chance of finding genic markers, but AFLP markers should still be obtained. The absence of deletions of genic markers in the early and intermediate-stage male steriles may indicate that the region containing the M1 and M2 loci is such a region. Regions also will not be ascertained if deletion causes loss of genes essential for viability of the pollen or the progeny of the irradiated plants. However, in *S. latifolia*, both the cytogenetic and anonymous marker data suggest that large Y chromosome regions, containing many markers, can be deleted without lethality (although their mortality rates cannot be estimated since the rate of deletion is unknown relative to the numbers of plants ascertained).

Conclusions: Our finding of presence/absence polymorphism for the *SlY6b* marker shows that deletions of Y regions exist in material sampled from wild populations. This is consistent with the previous finding that some wild-type Y chromosomes have detectable X43.1 sequences at only the PAR (*Yq*) end while others have them at both ends of the Y (Westergaard 1958; Farbos *et al.* 1999; Lardon *et al.* 1999). Thus Y chromosomes in this species are not uniform. It will therefore be important for future deletion mapping of this Y chromosome to ensure that the markers used are present in the parental strain used. This is likely to be particularly important for anonymous markers, such as AFLPs. Unfortunately, such information is not available for the markers that have so far been used. Clearly, if deletions arise at a high rate, and if the progeny of irradiated male plants (as previously assumed) have multiple deletions of different Y regions, this will make mapping the Y chromosome very difficult. It may be impossible to resolve the Y gene order without using genic markers in FISH experiments, but data using PCR should, when possible, be checked by sequencing to ensure that bands definitively identify the presence of the sequence.

Finally, if multiple rearrangements have occurred, it may be impossible to use X–Y divergence levels to localize regions of the Y chromosome that are likely to contain the sex-determining genes. The gene most often deleted along with the region containing the Su^F gene is *SlCypY*, which belongs to a stratum that formed much more recently than the time at which recombination was initially suppressed, while several Y-linked genes that belong to the stratum formed longest ago are not located near any sex-determining loci. The other well-defined region whose deletion affects the sex phenotype is the *p* arm region in which deletions cause late-stage male-sterility mutations with well-developed anthers, but pollen defects. Did this locus have a role in sex chromosome evolution? It is unlikely to be a primary sex-determining gene, but the Y-linked allele is probably not simply a gene required for male fertility that happens to be located on this chromosome. If that were true, there should be an X homolog and males with deletions in this region of the Y chromosome would be expected to have normal functions, unless loss of function of the gene is dominant or semidominant, which is unusual for such mutations (Zluvova *et al.* 2007).

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